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(71) Applicant: CHIRON CORPORATION [US/US]; 4560 Horton Street, Emeryville, CA 94608 (US).

(72) Inventor: GESNER, Thomas; 257 Trinity Avenue, Kensington, CA 94708 (US).

(74) Agents: SAVEREIDE, Paul, B. et al.; Chiron Corporation, Intellectual Property - R440, P.O. Box 8097, Emeryville, CA 94662-8097 (US).

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(54) Title: ANTIBODIES TO FAS ANTIGEN CAPABLE OF INHIBITING APOPTOSIS

(57) Abstract

Antibodies capable of recognizing and binding Fas antigen and further capable of inhibiting apoptosis, as well as hybridomas capable of producing the antibodies are disclosed. Methods for use of the antibodies to treat disease wherein apoptosis is implicated are also disclosed.

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ANTIBODIES TO FAS ANTIGEN CAPABLE OF INHIBITING APOPTOSIS

Technical Field

The invention relates to the field of immunology. More specifically, the invention relates to antibodies capable of binding Fas antigen and preventing apoptosis, to hybridomas capable of producing the antibodies and to methods of using the antibodies to treat and prevent diseases and symptoms of diseases caused by apoptosis.

Background of the Invention

The presence of Fas antigen on cell surfaces was first described by Yonehara et al, (1989) J. Exp. Med. 169:1747-1756. Fas antigen was described as a cell surface component with a molecular weight of approximately 200,000 daltons. Yonehara *et al* identifed Fas antigen by means a monoclonal antibody capable of binding to Fas antigen. The monoclonal antibody had an activity the authors described as "indistinguishable from the cytolytic activity of TNF." However, it was pointed out that the molecular weight of Fas antigen is different from that of the TNF receptor. Fas antigen appears on cells that do not express the TNF receptor and on cells that do express the TNF receptor. On those cells positive for Fas antigen and TNF receptor, Fas antigen is co-downregulated with the TNF receptor when the cells are incubated with TNF or with the anti-Fas antigen antibody.

Subsequently, the Fas antigen has been detected on the cell surface of myeloid cells, T lymphoblastoid cells and diploid fibroblasts. The cDNA for human Fas has been cloned and sequenced. Itoh *et al*, (1991) Cell 66:233-243. The sequence was also used to express Fas on the surface of murine T lymphoma WR19L and fibroblast L929 cells.

Binding of an anti-Fas antibody resulted in the death of these cells by apoptosis accompanied by fragmentation of chromosomal DNA and fragmentation of nuclei.

The Fas Ligand has been cloned by Suda *et al*, (1993) Cell 75:1169-1178 and the protein sequence determined from the nucleotide sequence reveals that Fas Ligand is a type II integral membrane protein with homology to tumor necrosis factor. Interaction of Fas antigen and Fas Ligand leads to apoptosis and is involved in T-cell mediated cytotoxicity.

Antibodies to Fas antigen are known in the art to induce apoptosis and have been administered to mice with resulting fulminating hepatitis due to apoptosis in liver cells. Ogaswara *et al*, (1993) Nature 364:806-809. Further, it is known that autoimmune diseases such as Sjögren syndrome, type I diabetes and viral hepatitis are associated with T-cell migration and infiltration of the affected area. Therefore, a need exists in the art for molecules capable of binding Fas and inhibiting apoptosis onset.

Brief Description of the Drawings

Figure 1 shows the results of experiments utilizing the antibodies of the invention. SKW 6.4 cells (2×10^4 cells) were preincubated with C33 (open triangle); C40 (closed square); C28 (closed triangle); or C42 (closed circle) for 1 hour at 37°C. CH11 was added to 100 ng/ml and incubated for 20 hours at 37°C. The last two hours in the presence of 1 μCi ^{3}H-thymidine. The results show that C28 and C42 antibodies are potent inhibitors of CH11 induced cell death across the range of concentrations tested. C33 and C40 were more effective at higher concentrations of antibody. The results of measurements of incorporation of ^{3}H-thymidine by SKW 6.4 cells with (lower arrow) and without (upper arrow) treatment with CH11 are shown on the left axis of the figure.

Figure 2 shows the inhibition of Fas ligand killing of SKW 6.4 cells by monoclonal antibodies. SKW 6.4 cells (2.86×10^4 cells) were preincubated with C33 (open square); C40 (open diamond); C28 (closed diamond); or C42 (closed square) for 1 hour at 37°C. SF9 cells expressing Fas ligand on the cell surface (5.72 x 103) were added to the mixture resulting in E:T = 0.2:1. The cells were then co-cultured for 6 hours at 37°C. The last 2.5 hours in the presence of 1 μCi ^{3}H-thymidine. The results show that that all of the antibodies tested are capable of inhibiting apoptosis.

Figure 3 shows that the antibodies of the invention are capable of blocking the interaction of cells expressing Fas antigen with cells expressing Fas ligand on their respective cell surfaces. SKW 6.4 cells were preincubated with and without antibody and co-cultured with SF9 cells expressing Fas ligand on the cell surface, essentially as describe in Figure 2. Figure 3A is a photomicrograph of a co-culture without antibody. Multiple rosettes of SF9 (larger) and SKW 6.4 (smaller) cells may be seen indicating cell-cell interactions. Figure 3B is a photomicrograph of a co-culture with antibody and no rosettes are seen.

Figure 4 shows the inhibition of Fas ligand killing of SKW 6.4 cells cells were preincubated with C42 or ZB.4 (Immunotech, Westbrook ME) and co-cultured with Sf9 cells expressing Fas ligand on the cell surface essentially as described above. Thymidine incorporation is shown for C42 (open squares) and ZB.4 (closed squares). Incorporation of thymidine for SKW 6.4 cells alone (upper arrow) and for SKW 6.4 cells co-cultured with Sf9-Ligand cells (lower arrow) are shown on the right vertical axis.

Brief Description of the Invention

The invention relates to hybridomas capable of producing antibodies capable of recognizing and bind Fas antigen and to inhibit apoptosis upon binding. The invention also relates to the antibodies produced by the hybridomas. Finally, the invention relates to methods of treatment using the antibodies.

Detailed Description of the Invention

As used herein, the term "Fas antigen" refers to the cell surface antigen cloned by Itoh *et al*, (1991) Cell 66:233-243. The Fas antigen is approximately 200,000 MW and can mediate cell death by apoptosis.

As used herein, the term "Fas Ligand" refers to the cell surface protein cloned by Suda *et al*, Cell (1993) 75:1169-1178. Fas Ligand interacts with Fas antigen and induces apoptosis in the cell having Fas antigen on its surface.

As used herein, the term antibody encompasses monoclonal antibodies and fragments thereof. Such fragments will include Fab, Fab2, Fv. Antibodies should also be understood to include humanized antibodies. Such antibodies are designed to reduce immunogenicity of the antibodies in human patients and therefore mitigate any HAMA response. Humanization may be accomplished, for example, by a CDR grafting approach in which CDR regions are inserted within a set of human framework regions, or by a veneering approach in which amino acids exposed at the surface of the folded antibody protein are replaced with consensus amino acids from the human framework regions at the same positions (see EP 519,596, published 23 December 1992).

Example

A. Cloning / Expression / Purification of Soluble Fas antigen (sFas)

1. Total RNA isolation from Fas^+ cells

Cells expressing Fas antigen on their cell surface (Fas^+ cells) (e.g. U937 cells) are solubilized in guanidine isothiocyanate and fractionated over cesium chloride. The RNA pellet may then be solubilized in water and the total RNA preparation incubated with reverse transcriptase to synthesize cDNA. Sambrook *et al.* Molecular Cloning: A Laboratory Manual (2nd Edition, 1989). Maniatis *et al.* Molecular Cloning: A Laboratory Manual (1982).

2. PCR of cDNA: Engineering of sFas

Using PCR primers based on the human Fas published sequence (Itoh *et al*, (1991) Cell 66:235-243), cDNA encoding the extracellular region of Fas with a C-terminus epitope tag (glu-glu) (sFas) may be constructed. The 5' primer may utilize a sequence 5' of the initiating ATG codon preceded by an insertion of a sequence encoding for a Pst I restriction enzyme site. The 3' primer may consist of a coding region 5' of ASN^{157}, including the ASN^{157} sequence, followed by the glu-glu sequence, a stop codon and a Not I restriction enzyme site sequence. The sequence of the 5' and 3' primers may be: 5'-GTACCTGCAGGGAAGCTCTTTCACTTCGGAGG-3 and 5'-GTACGCGGCCGCTTATTCCATTGGCATGTATTCGTTAGATCTGGATCCTTCCT CTTTGC-3', respectively. The PCR reaction is carried out under appropriate conditions.

3. Ligation into pAcC13 expression vector

Both the baculovirus expression plasmid pAcC13 and PCR product are digested with Pst I and Not I. The plasmid may be further treated with calf intestine alkaline

phosphatase. The digested plasmid and PCR product are purified (Glassmilk, BIO-101), and subsequently ligated with T4 ligase.

Escherichia coli (*E. coli*) are transformed with the products of the ligase reaction. Positive colonies (i.e. those colonies containing plasmid with insert) are further screened by PCR using the cloning primers.

4. Baculovirus/Sf9 expression

Using standard protocols and/or commercially available expression systems, the cDNA for sFas may be incorporated into baculovirus for expression of sFas in Sf9 cells (*Spodoptera frugiperda*). Expression in insect cells has also been described by Summers and Smith (1987) *Texas Agricultural Experiment Station Bulletin No. 1555.* Commercially available kits include the MaxBac kit (Invitrogen, San Diego, CA). The baculovirus infected Sf9 cells lead to the expression and secretion of sFas into culture medium.

5. Affinity purification of sFas

sFas contained in Sf9 medium is concentrated over a YM10 membrane (Amicon), then passed over an anti-glu-glu antibody column (anti-glu-glu monoclonal antibody coupled to protein G Sepharose). Bound sFas is eluted with the glu-glu hexapeptide. After separation of the hexapeptide from sFas, the homogeneous sFas is used as antigen for monoclonal antibody production.

6. Production of Sf9 cells expressing Fas-Ligand on the cell surface

Sf9 cells capable of expressing full length Fas-Ligand on the cell surface may be developed essentially as described above, using the published sequence of Fas-Ligand

(Suda *et* al, (1993) Cell 75:1169-1178). It will be appreciated that the addition of a glu-glu epitope tag is unnecessary.

B. Production of Hybridomas Capable of Producing Fas Monoclonal Antibody

sFas antigen produced by expression in SF9 cells was used as an immunogen in mice to produce hybridomas capable of producing antibodies which recognize and bind Fas antigen. The method used was essentially that of Kohler and Milstein, (1975) Nature 256:495-497 with a standard PEG fusion modification. Although Fas antigen produced by in SF9 cells was used as an immunogen, Fas antigen from other sources may also be used. Such sources include recombinant Fas antigen produced in E. coli and yeast such as *Saccharomyces cerevisiae*. Cells expressing Fas antigen on their surface may also be used as immunogen, including SKW 6.4 and U937 cells. SF9 cells capable of expressing Fas antigen on the cell surface may also be prepared as described in U.S. 5,397,703, the disclosure of which is herein incorporated by reference.

Primary screens of hybridomas for antibodies capable of binding Fas antigen may be conducted by Fas ELISA. The Fas ELISA consists essentially of coating Fas antigen (100 ng or more/well) onto a 96 well plate, blocking said wells with an irrelevant protein (e.g. bovine serum albumin) and incubating with hybridoma supernatants. The wells are then incubated with an enzyme conjugated anti-mouse antibody (e.g. alkaline phosphatase or horse radish peroxidase - coupled to goat, rabbit, donkey or sheep anti mouse immunoglobulin) and develeoped with an appropriate substrate. The wells are read on an ELISA reader (e.g. Dynatech MRX plate reader).

C. Further Screening of Monoclonal Antibodies for Anti-Apoptosis Activity

Using the methods described above, ten hybridomas were identified and subjected to further analysis.

1. Inhibition of Apoptosis in a Fas^+ cell line

SKW6-4 cells (human B cell line, Fas^+) were pre-incubated with $ELISA^+$ monoclonal antibodies. Cells expressing Fas ligand on the cell surface ($Fas\text{-}L^+$ cells) or an apoptosis- inducing antibody (e.g. CH11, Immunotech) were added and co-cultured for 5-24 hrs at 37°C, the last two in the presence of ^3H-thymidine (1 μCi). Cells were lysed and DNA collected using a SKATRON cell harvester. Radioactivity was enumerated in a liquid scintillation counter. Enhanced ^3H-thymidine incorporation caused by the monoclonal antibody induced suppression of Fas-L mediated cell death identified which monoclonal antibodies had anti-apoptosis activity.

Figure 1 shows the results of experiments utilizing four antibodies of the invention. SKW 6.4 cells (2×10^4 cells) were preincubated with C33-1.6.1 (C33) (open triangle); C40-1.3.3 (C40) (closed square); C28-3.7.1 (C28) (closed triangle); or C42-7.4.5 (C42) (closed circle) for 1 hour at 37°C. CH11 (apoptosis - inducing antibody) was added to 100 ng/ml and incubated for 20 hours at 37°C. The last two hours in the presence of 1 μCi ^3H-thymidine. The results show that C28 and C42 antibodies are potent inhibitors of CH11 induced cell death across the range of concentrations tested. C33 and C40 were more effective at higher concentrations of antibody. Their respective isotypes of the four antibodies are C28:IgG_1, C33:IgG_1, C40:IgG_{2b} and C42:IgG_1. The results of measurements of incorporation of ^3H-thymidine by SKW 6.4 cells with (lower

arrow) and without (upper arrow) treatment with CH11 are shown on the left axis of the figure.

2. Prevention of Fas-Fas-L Interaction

Figure 2 shows the inhibition of Fas ligand killing of SKW 6.4 cells by the monoclonal antibodies of the invention. SKW 6.4 cells (2.86×10^4 cells) were preincubated with C33 (open square); C40 (open diamond); C28 (closed diamond); or C42 (closed square) for 1 hour at 37°C. SF9 cells expressing Fas ligand on the cell surface (5.72 x 103) were added to the mixture resulting in E:T = 0.2:1. The cells were then co-cultured for 6 hours at 37°C. The last 2.5 hours in the presence of 1 μCi ^{3}H-thymidine. The results show that that all of the antibodies tested are capable of inhibiting apoptosis.

Figure 4 shows the inhibition of Fas ligand killing of SKW 6.4 cells cells were preincubated with C42 or ZB.4 (Immunotech, Westbrook ME) and co-cultured with Sf9 cells expressing Fas ligand on the cell surface essentially as described above. Thymidine incorporation is shown for C42 (open squares) and ZB.4 (closed squares). Incorporation of thymidine for SKW 6.4 cells alone (upper arrow) and for SKW 6.4 cells co-cultured with Sf9-Ligand cells (lower arrow) are shown on the right vertical axis.

Figure 3 shows that the antibodies of the invention are capable of blocking the interaction of cells expressing Fas antigen with cells expressing Fas ligand on their respective cell surfaces. SKW 6.4 cells were preincubated with and without antibody and co-cultured with SF9 cells expressing Fas ligand on the cell surface, essentially as describe in Figure 2. Sf9/Fas-L expressing cells were added and the mixture was centrifuged (1500 rpm, 2 minutes) and incubated 1-3 hrs at 25°C-27°C. The cell pellet was gently

resuspended and resolved by photomicroscopy. Figure 3A is a photomicrograph of a co-culture without antibody. Multiple rosettes of SF9 (larger) and SKW 6.4 (smaller) cells may be seen indicating cell-cell interactions. Figure 3B is a photomicrograph of a co-culture with antibody and no rosettes are seen.

Deposit Information

The following materials were deposited with the American Type Culture Collection:

Hybridoma	Deposit Date	CMCC Acc. No.	A.T.C.C. Acc. No.
C33-1-6-1,	June 07, 1995	CMCC #11263;	
C40-1.3.3,	June 07, 1995	CMCC #11264;	
C28-3-7-1,	June 07, 1995	CMCC #11266;	
C42-7-4-5,	June 07, 1995	CMCC #11267	

The above materials were deposited by Chiron Corporation, an assignee of the present invention with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for purposes of Patent Procedure. The accession number is available from the ATCC at telephone number (301) 881-2600. The Chiron Master Culture Collection Accession Number is also provided.

These deposits are provided as convenience to those of skill in the art, and are not an admission that a deposit is required under 35 U.S.C. §112. The nucleic acid sequence of these deposits, as well as the amino acid sequence of the polypeptides encoded thereby, are incorporated herein by reference and should be referred to in the event of an error in

the sequence described herein. A license may be required to make, use, or sell the deposited materials, and no such license is granted hereby.

Claims

I claim:

1. A murine x murine hybridoma selected from the group consisting of:

(a) C42-7-4-5;

(b) C28-3-7-1;

(c) C40-1.3.3; and

(d) C33-1-6-1.

2. An antibody or fragment thereof, said antibody or fragment thereof capable of recognizing and binding Fas antigen, and further capable blocking the binding of a monoclonal antibody, said monoclonal antibody produced by a murine x murine hybridoma selected from the group consisting of:

(a) C42-7-4-5;

(b) C28-3-7-1;

(c) C40-1.3.3; and

(d) C33-1-6-1.

3. The antibody or fragment thereof of claim 2 wherein the antibody is a humanized antibody.

4. A method of treating a disease, said disease characterized by increased apoptosis, comprising administration of a monoclonal antibody to a patient in need of such treatment, said monoclonal antibody produced by a murine x murine hybridoma selected from the group consisting of:

(a) C42-7-4-5;

(b) C28-3-7-1;

(c) C40-1.3.3; and

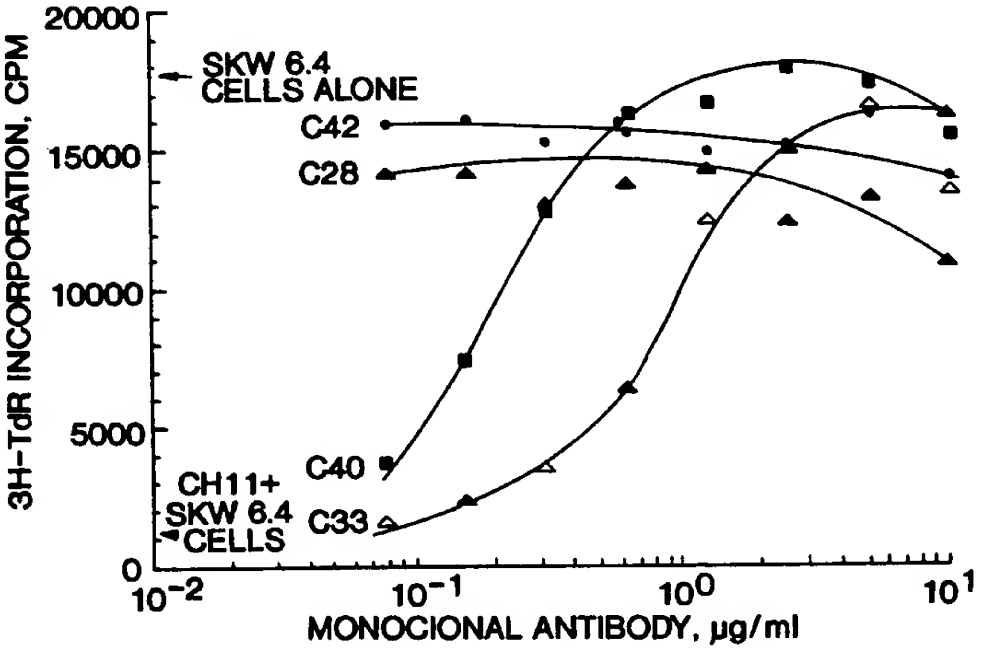
(d) C33-1-6-1.

5. The method of claim 4, wherein the disease is rheumatoid arthritis.

6. The method of claim 4, wherein the disease is Sjögren syndrome.

7. The method of claim 4, wherein the disease is type I diabetes or diabetes mellitus.

8. The method of claim 4, wherein the disease is viral hepatitis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CELLS ALONE
C42
C28
C40
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C33
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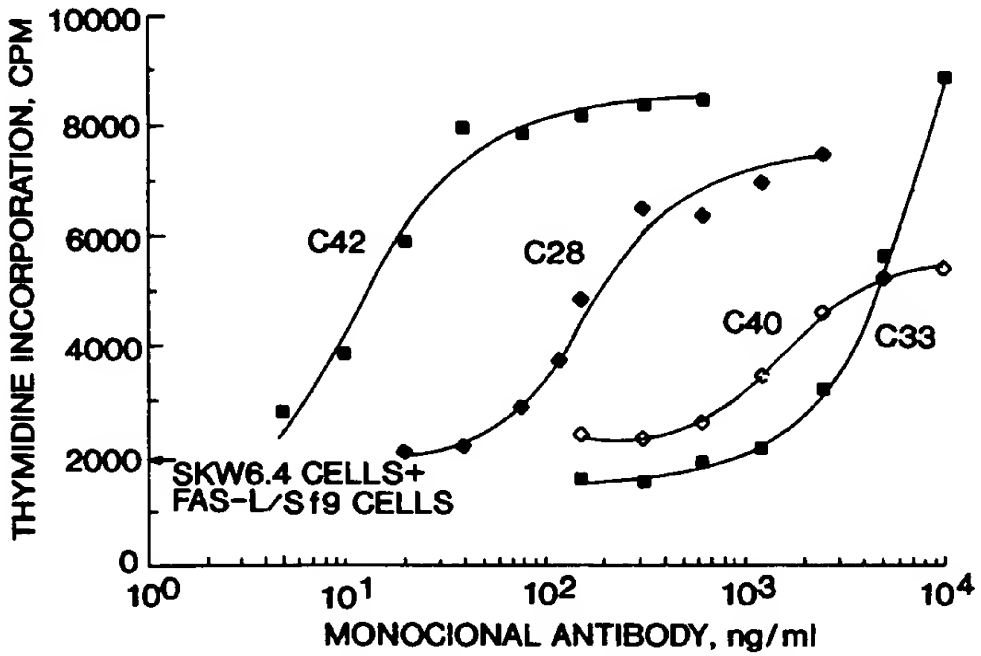
10^{1}
MONOCIONAL ANTIBODY, µg/ml

FIG. 1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C33
SKW6.4 CELLS+
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10^{3}
10^{4}
MONOCIONAL ANTIBODY, ng/ml

FIG. 2

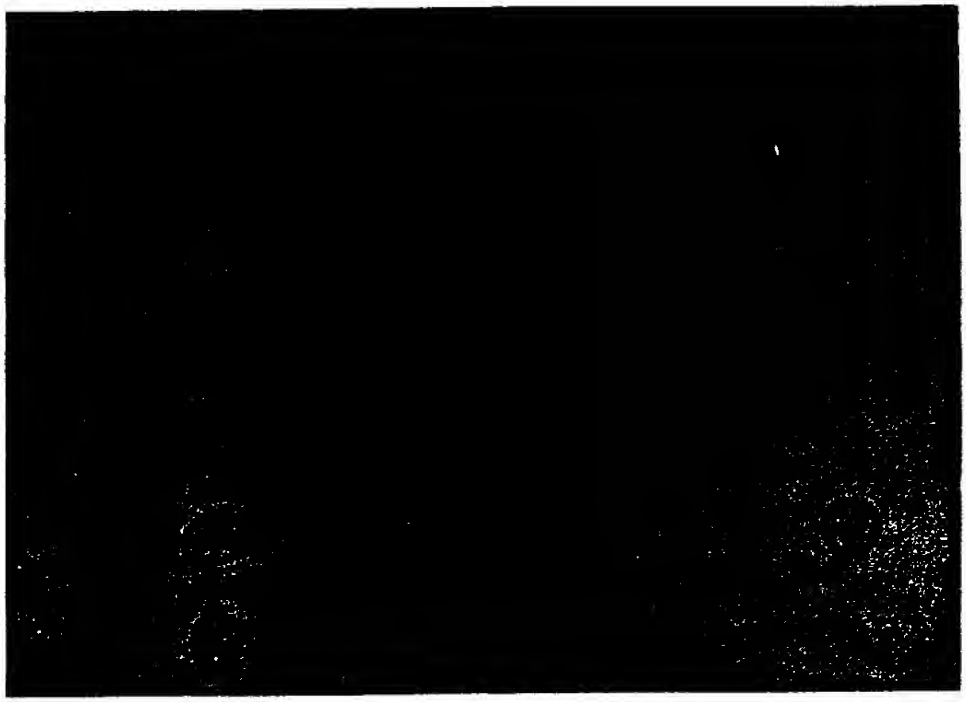


FIG. 3A

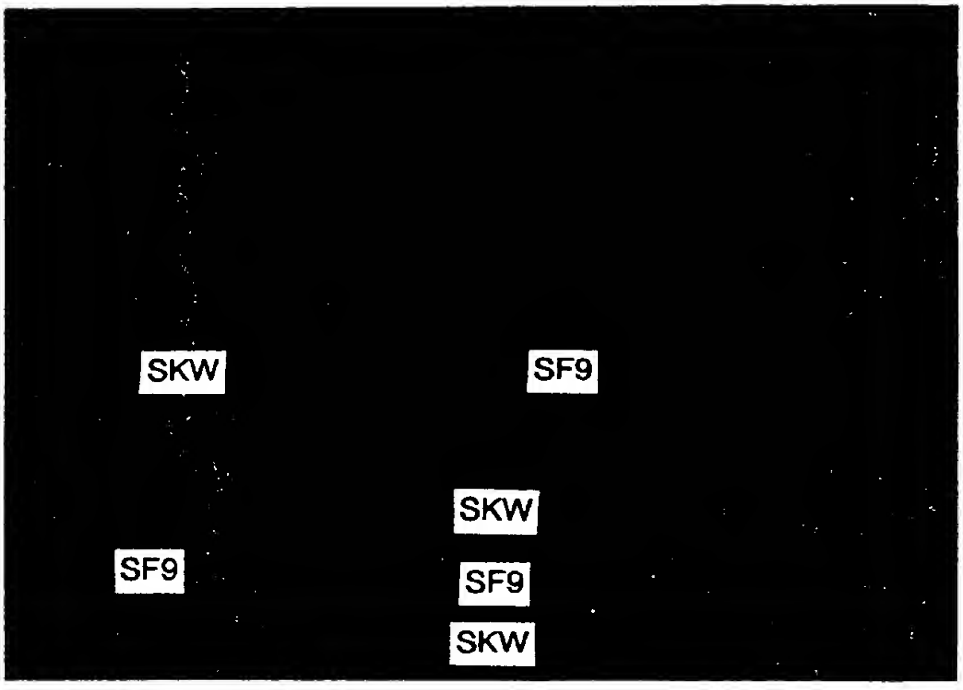
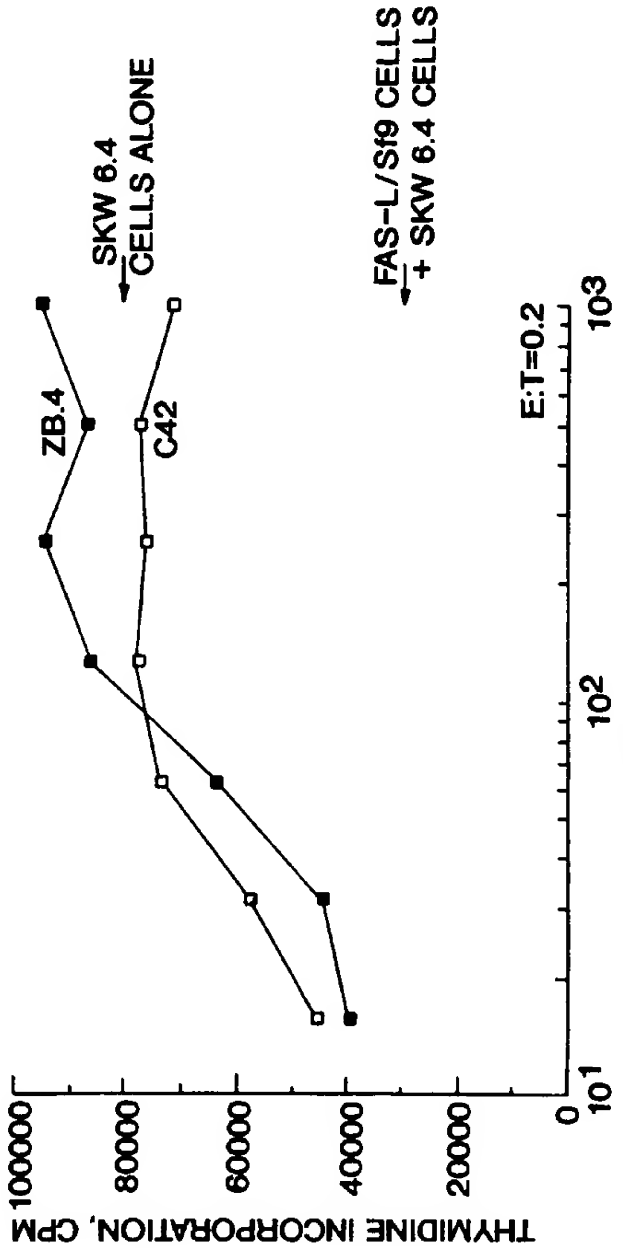


FIG. 3B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M'Ab, µg/ml
E:T=0.2
ZB.4
C42
SKW 6.4
CELLS ALONE
FAS-L/Sf9 CELLS
+ SKW 6.4 CELLS

FIG. 4

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(74) Agents: SAVEREIDE, Paul, B. et al.; Chiron Corporation, Intellectual Property - R440, P.O. Box 8097, Emeryville, CA 94662-8097 (US).

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 96/09153

A. CLASSIFICATION OF SUBJECT MATTER
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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 10540 A (IMMUNEX CORP.) 20 April 1995 see the whole document ---	2-5
X	VIROLOGY, vol. 209, no. 2, 1 June 1995, SAN DIEGO, CA, USA, pages 288-296, XP002024489 T. TAKIZAWA ET AL.: "Activation of the apoptotic Fas antigen-encoding gene upon influenza virus infection involving spontaneously produced beta-interferon." see abstract --- -/--	2,4

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
4 February 1997	12.02.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Nooij, F

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/US 96/09153

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	INTERNATIONAL IMMUNOLOGY, vol. 6, no. 11, November 1994, OXFORD, GB, pages 1799-1806, XP000616652 M. ALDERSON ET AL.: "Regulation of apoptosis and T cell activation by Fas-specific mAb." see the whole document	2,4
X	INTERNATIONAL IMMUNOLOGY, vol. 6, no. 12, December 1994, OXFORD, GB, pages 1849-1856, XP000616653 S. YONEHARA ET AL.: "Involvement of apoptosis antigen Fas in clonal deletion of human thymocytes." see abstract see page 1853, left-hand column, line 28 - right-hand column, line 29	2,4
A	THE JOURNAL OF IMMUNOLOGY, vol. 149, no. 10, 15 November 1992, BALTIMORE, MD, USA, pages 3166-3173, XP002024490 J. DHEIN ET AL.: "Induction of apoptosis by monoclonal antibody anti-APO-1 class switch variants is dependent on cross-linking of Apo-1 cell surface antigens." see abstract	1-8
A	WO 91 10448 A (GERMAN CANCER RESEARCH CENTER) 25 July 1991 see claims	1-8
P,X	DE 44 47 484 A (DEUTSCHES KREBSFORSCHUNGSZENTRUM STIFTUNG DES ÖFFENTLICHEN RECHTS) 26 October 1995 see example 2 see claims	2,4

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

ternational application No.
PCT/US 96/09153

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 4-8
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 4-8 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 96/09153

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9510540	20-04-95	AU-A-	7978494	04-05-95
		CA-A-	2174132	20-04-95
		EP-A-	0723556	31-07-96
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		DE-D-	69009497	07-07-94
		DE-T-	69009497	24-11-94
		EP-A-	0511202	04-11-92
DE-A-4447484	26-10-95	DE-C-	4412177	02-11-95
		WO-A-	9527735	19-10-95
		EP-A-	0705278	10-04-96
		JP-T-	8511692	10-12-96